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# Copy number loss in the region of the *ASPN* gene in patients with acetabular dysplasia

*ASPN* CNV IN ACETABULAR DYSPLASIA

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## Objectives

We have previously investigated an association between the genome copy number variation (CNV) and acetabular dysplasia (AD). Hip osteoarthritis is associated with a genetic polymorphism in the aspartic acid repeat in the N-terminal region of the asporin (*ASPN*) gene; therefore, the present study aimed to investigate whether the CNV of *ASPN* is involved in the pathogenesis of AD.

## Methods

Acetabular coverage of all subjects was evaluated using radiological findings (Sharp angle, centre-edge (CE) angle, acetabular roof obliquity (ARO) angle, and minimum joint space width). Genomic DNA was extracted from peripheral blood leukocytes. Agilent's region-targeted high-density oligonucleotide tiling microarray was used to analyse 64 female AD patients and 32 female control subjects. All statistical analyses were performed using EZR software (Fisher's exact probability test, Pearson's correlation test, and Student's *t*-test).

## Results

CNV analysis of the *ASPN* gene revealed a copy number loss in significantly more AD patients (9/64) than control subjects (0/32;  $p = 0.0212$ ). This loss occurred within a 60 kb region on 9q22.31, which harbours the gene for *ASPN*. The mean radiological parameters of these AD patients were significantly worse than those of the other subjects (Sharp angle,  $p = 0.0056$ ; CE angle,  $p = 0.0076$ ; ARO angle,  $p = 0.0065$ ), and all nine patients required operative therapy such as total hip arthroplasty or pelvic osteotomy. Moreover, six of these nine patients had a history of operative or conservative therapy for developmental dysplasia of the hip.

## Conclusions

Copy number loss within the region harbouring the *ASPN* gene on 9q22.31 is associated with severe AD. A copy number loss in the *ASPN* gene region may play a role in the aetiology of severe AD.

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## Article focus

- An association between acetabular dysplasia (AD) and chromosomal structural rearrangements has only been investigated in our previous study.
- We conducted a copy number variation (CNV) analysis of the *ASPN* gene region in cases with varying degrees of acetabular coverage.
- We investigated the association between the CNV of the *ASPN* gene and AD to

identify a possible genetic structural variation that confers a risk of AD.

## Key messages

- A significant loss of copy number was detected in the *ASPN* gene region in AD patients.
- The *ASPN* gene copy number loss may be an independent risk factor for developing AD, and could be a predictive tool to identify severe AD patients.

## Strengths and limitations

- This is the first report on the association of the CNV of the *ASPN* gene region with AD.
- The *ASPN* gene copy number loss showed significant association with severe AD.
- Elucidation in different ethnicities in larger sets of patients and controls will be warranted for validation of the findings in the near future.

## Introduction

Acetabular dysplasia (AD) is a major and common cause of secondary hip osteoarthritis (OA), and is a multifactorial disease that may involve both genetic and environmental factors. AD is characterised by a shallow hip socket and decreased coverage of the femoral head, which predisposes the hip joints to progressive damage of the cartilage, leading to OA. A nationwide epidemiological study of hip OA in Japan showed that the aetiology of hip OA was AD in 390 of 485 patients (80.4%), and the relative frequency of AD among hip OA patients was significantly greater in women (361; 92.6%) than in men (29; 7.4%).<sup>1</sup>

AD is associated with abnormally high stress on the anterolateral part of the acetabulum, which leads to degeneration of the articular cartilage. This can cause hip pain and dysfunction sufficient for the patient to require medical evaluation and treatment. The severity of AD ranges from barely detectable to severe hip malformation or dislocation. The pathogenesis of AD remains unclear, but it appears to be related to several different factors such as racial background, gender, and intrauterine positioning.<sup>1,2</sup> Few studies have investigated the association between genetic variation and AD.<sup>3-5</sup>

Recently, copy number variations (CNVs) have been recognised as inter-individual genetic variations.<sup>6</sup> CNVs represent structural rearrangements of the genome such as deletion or gain (including duplication and triplication). Sporadic diseases could be caused by different types of mutations, such as point mutations and genomic rearrangements. Several CNV abnormalities are significant factors in the aetiology of various diseases such as Charcot-Marie-Tooth disease, X-linked disease, and Smith-Magenis syndrome.<sup>7</sup> To date, an association between CNV and AD has only been examined in our previous study.<sup>8</sup> In this study we conducted a whole-genome CNV analysis, and identified a copy number loss within the region harbouring the *Semaphorin 4D* gene in 18 of 64 AD patients.

Asporin (*ASPN*) is an extracellular matrix protein that contains a unique aspartic acid (D) repeat in the N-terminal region.<sup>9,10</sup> *ASPN* belongs to the small leucine-rich proteoglycan (SLRP) family; members of the SLRP family bind to transforming growth factor beta (TGF- $\beta$ ), a key growth factor in cartilage metabolism, and to other extracellular matrix molecules of cartilage, including collagens.<sup>11</sup> *ASPN* mRNA is expressed abundantly in OA articular cartilage,<sup>9</sup> and previous studies have implicated

SLRP family genes in the aetiology of OA.<sup>12,13</sup> Kizawa et al<sup>14</sup> reported a significant association between a polymorphism in the D repeat of the gene encoding *ASPN* and OA in the Japanese population. However, the potential association between AD and chromosomal structural rearrangements of the *ASPN* gene region has not yet been studied.

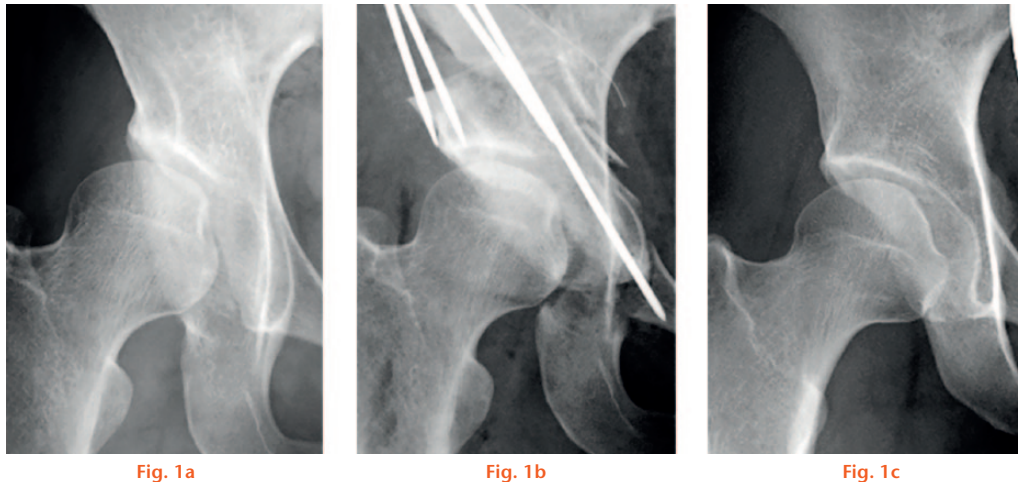
In the present study, we investigated the association between CNV and AD to identify a possible gross genetic structural variation that confers a risk of AD. We conducted a CNV analysis of the *ASPN* gene region using genomic DNA extracted from peripheral blood of subjects with varying degrees of acetabular coverage.

## Materials and Methods

**Sample size calculation.** A sample size calculation was performed using EZR software version 1.27 (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics.<sup>15</sup> The sample size calculation was based on a type I error probability of 0.05 (two-sided), 70% power, an assumed *ASPN* copy number loss rate of 0.3 for AD, and a minimal detectable *ASPN* CNV of 0.05. The study was powered to detect a change in the anticipated *ASPN* copy number loss rate of 0.3 for AD, as estimated on the basis of previous reports for CNV on sporadic diseases.<sup>8,16-18</sup> According to the sample size calculation for this case-control design study, the minimum sample sizes required were 58 evaluable patients and 29 controls.

**Study subjects and radiographs.** We recruited 64 consecutive unrelated Japanese female patients diagnosed with AD and 32 healthy female volunteers from our hospital during the same period. A total of 96 subjects living in our city or neighbouring areas were studied. All participants gave written informed consent for the genetic analysis, and this study was approved by the medical ethics committees of our research facility.

In this study, all subjects were Japanese females who underwent clinical, radiological, and blood examinations by orthopaedic specialists. To exclude other diseases such as inflammatory arthritis (rheumatoid, polyarthritic, or autoimmune disease), post-traumatic or post-septic arthritis, and a definitely positive Mendelian family history, all subjects completed a standard questionnaire regarding their medical history. All AD cases were sporadic in nature. Case status was determined according to the examination data and symptoms. Some individuals were undergoing medical treatment and/or had received operative therapy for AD (Fig. 1b). The control group had no signs or symptoms of arthritis or joint disease (pain, swelling, tenderness, or restriction of movement). No subjects dropped out during the process of the study.



Anteroposterior radiographs of acetabular dysplasia (AD) pre- and post-operatively, and a control hip: a) case 61: AD hip of a 42-year-old female; Sharp angle 56.3°; centre-edge (CE) angle 3.5°; acetabular roof obliquity (ARO) angle 29.6°; minimum joint space width (MJSW) 3.7 mm; developmental dysplasia of the hip, positive; b) case 61: post-periacetabular osteotomy; c) control 26: normal hip of a 30-year-old female; Sharp angle 38.6°; CE angle 28.8°; ARO angle 9.9°; MJSW 3.3 mm.

The respective mean ages of the 64 AD patients and 32 control subjects were 51.3 years (SD 15.2, 21 to 75) and 35.5 years (SD 9.5, 29 to 68).

The evaluation of acetabular coverage was based on radiological findings. Radiographs were taken with a tube-to-film distance of 100 cm. The pelvises were in a supine position with the frontal anatomical plane parallel to the film plate. The central beam was directed at the midpoint between the pubic symphysis and a horizontal line connecting bilateral anterior superior iliac spines. All radiographs fulfilled the criteria for correct pelvic positioning regarding the axial and transverse pelvic rotation. The Sharp angle was defined as the angle formed between a line from the inferior pelvic teardrop to the superior lateral edge of the acetabulum and a line horizontal from the inferior pelvic teardrop.<sup>19</sup> The centre-edge (CE) angle was defined as the angle formed by a vertical line and a line connecting the femoral head centre with the lateral edge of the acetabulum.<sup>20</sup> The acetabular roof obliquity (ARO) angle was defined as the angle formed by a horizontal line and a line connecting the medial point of the sclerotic zone with the lateral edge of the acetabulum.<sup>21</sup> The minimum joint space width (MJSW) was defined as the shortest distance between the femoral head margin and the acetabulum in the weight-bearing zone, as defined by the medial and lateral aspects of the acetabular sourcil.<sup>22</sup> The diagnosis of AD was based on the presence of one of the following radiological findings: Sharp angle  $\geq 42^\circ$ , CE angle  $\leq 25^\circ$ , or ARO  $\geq 15^\circ$  on at least one side (Fig. 1a). The radiological parameters in each case were independently measured twice by two authors (TS and EC). We could not evaluate the CE angle and MJSW in case 39 as it had a bilaterally high congenital dislocation of the hip.

We analysed the high-density oligonucleotide array using the genomic DNA extracted from peripheral blood leukocytes of the 64 female AD patients, and also that of the 32 female control subjects with Sharp angle  $< 42^\circ$ , CE angle  $> 25^\circ$ , and ARO  $< 15^\circ$  on both sides (Fig. 1c).

**High-density custom-made oligonucleotide tiling microarray.** Agilent's high-density oligonucleotide tiling microarray assay (Agilent Technologies, Santa Clara, California) was performed on a custom-designed microarray that targeted the 60 kb *ASPN* gene region on 9q22.31 (Chr9:94 250 000 to 94 310 000; NCBI Build 36.1, hg18), as described previously.<sup>23</sup> Data analysis of the microarray experiments was conducted using the Aberration Detection Method-2 statistical algorithm (Agilent Technologies), based on the combined log<sub>2</sub> ratios at a threshold of five, as was undertaken in our previous study.<sup>8</sup> The data were centralised, and calls with average log<sub>2</sub> ratios  $< 0.15$  were filtered to exclude false positives. Abnormal copy number, losses and gains, in a complex multi-copy variable region by high-density tiling array, were identified by deviation of probe log<sub>2</sub> ratios that exceeded a given threshold of 1.5 SD from the mean probe ratio.<sup>24,25</sup>

**Statistical analysis.** All numerical values are expressed as the mean and SD. Statistical analyses of the clinical data were performed with Fisher's exact probability test, Pearson's correlation test, and Student's *t*-test. Values of  $p < 0.05$  were considered statistically significant. All statistical analyses were performed using EZR software version 1.27 (Saitama Medical Center, Jichi Medical University, Saitama, Japan).

## Results

**Radiological parameters of the acetabular dysplasia patients and control subjects.** The mean radiological parameters on

**Table I.** Mean radiological parameters in the acetabular dysplasia and control groups

| Radiological parameter | AD group (n = 64)          | Control group (n = 32)   | p-value (t-test) |
|------------------------|----------------------------|--------------------------|------------------|
| Sharp angle (°)        | 51.9 (3.5; 44.5 to 60.3)   | 39.5 (2.0; 34.0 to 41.6) | 7.58E-33         |
| CE angle (°)           | 3.1 (11.9; -24.5 to 23.9)* | 32.9 (4.1; 26.1 to 42.8) | 4.52E-24         |
| ARO angle (°)          | 29.7 (8.0; 7.7 to 51.5)    | 4.5 (4.0; -3.9 to 12.0)  | 6.87E-30         |
| MJSW (mm)              | 2.5 (1.8; 0 to 7.3)*       | 3.9 (0.6; 3.3 to 5.5)    | 8.12E-5          |

\*case 39 had a bilaterally high congenital dislocation of the hip and was excluded

Values on the worst side are presented as mean (SD; range)

AD, acetabular dysplasia; CE, centre edge; ARO, acetabular roof obliquity; MJSW, minimum joint space width

**Table II.** Correlation coefficient between radiological parameters in all subjects

| Radiological parameter (n = 95)* | Correlation coefficient | p-value (Pearson's correlation) |
|----------------------------------|-------------------------|---------------------------------|
| Sharp angle to CE angle          | -0.810                  | 3.13E-23                        |
| Sharp angle to ARO angle         | 0.867                   | 6.76E-30                        |
| Sharp angle to MJSW              | -0.300                  | 0.003                           |
| CE angle to ARO angle            | -0.893                  | 5.43E-34                        |
| CE angle to MJSW                 | 0.434                   | 1.12E-5                         |
| ARO angle to MJSW                | -0.387                  | 0.0001                          |

\*case 39 had a bilaterally high congenital dislocation of the hip and was excluded

CE, centre edge; ARO, acetabular roof obliquity; MJSW, minimum joint space width

the worst side of the AD patients and the control subjects are shown in Table I. These radiological parameters correlated significantly with each other (Table II).

**High-density tiling microarray analysis of the acetabular dysplasia patients and control subjects.** We analysed the extent of the copy number change along the 60 kb genomic region of 9q22.31 harbouring the *ASPN* gene using a custom-made high-density oligonucleotide tiling microarray. A loss of copy number was found in nine of the 64 AD patients, but in none of the 32 control subjects (9/64 vs 0/32,  $p = 0.0212$ , Fisher's exact probability test). Figure 2 shows the detailed structure of the segmental loss in nine AD patients and two controls along the 60 kb region (AD patients: Figs 2a to 2i; control subjects: Figs 2j and 2k). Figure 3 shows a schematic of the extent of the loss of copy number in the *ASPN* gene region in all nine of the AD patients who exhibited loss. In cases 4, 20, 39, 55, and 61, the range of the loss covered the upper side of the *ASPN* gene, including the *ASPN* D repeat region in exon 2; in the other cases, the range covered the whole *ASPN* gene (Fig. 3).

**Characteristics of the acetabular dysplasia patients with positive copy number loss in the *ASPN* gene.** The Sharp angle, CE angle, and ARO angle in the AD patients with loss of copy number and in the other 87 subjects with no loss in the *ASPN* gene are shown in Figure 4. The Sharp angle in the nine AD patients with loss was  $53.6^\circ$  (SD  $3.6^\circ$ ), which was significantly larger than that in the 87 subjects that had no loss ( $47.2^\circ$ , SD  $6.6^\circ$ ;  $p = 0.0056$ , Student's *t*-test). The CE angle in the eight AD patients with loss was  $-2.3^\circ$  (SD  $12.9^\circ$ ), which was significantly smaller than that in the 87 subjects with no loss ( $14.6^\circ$ , SD  $17.0^\circ$ ;  $p = 0.0076$ , *t*-test). The ARO angle in the nine

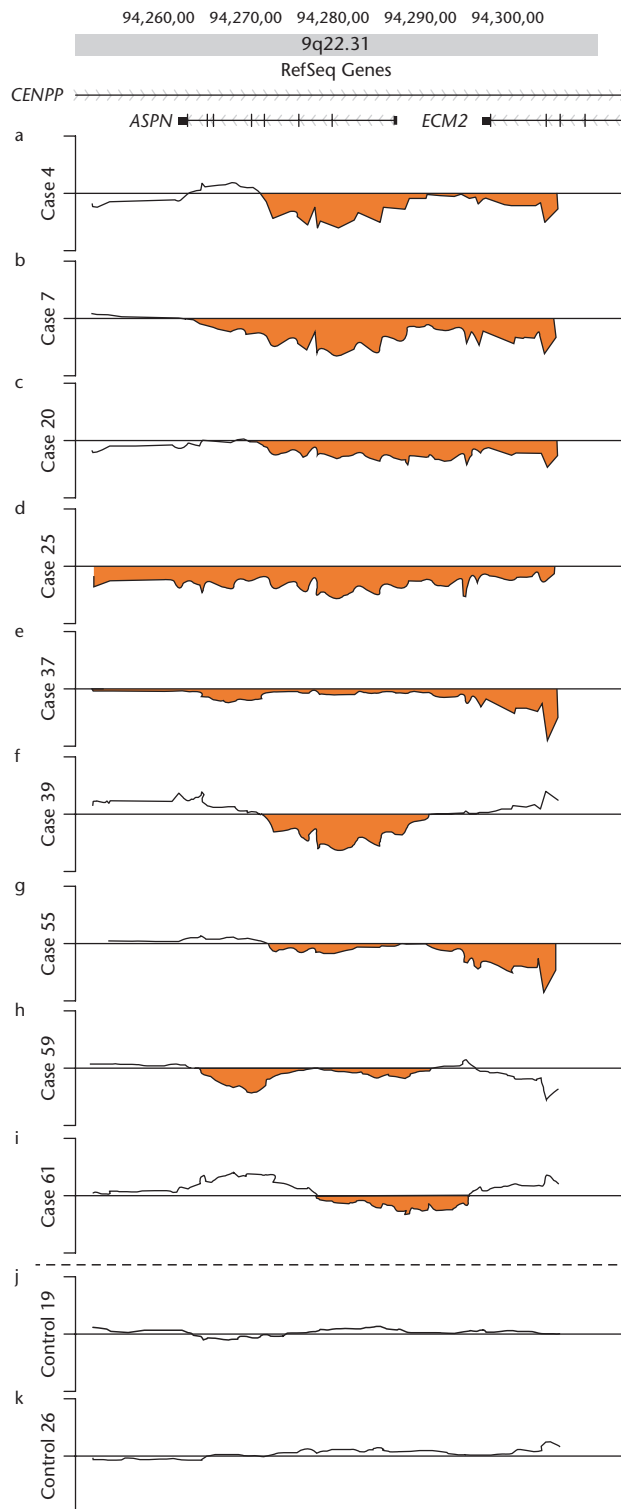
AD patients with loss was  $33.1^\circ$  (SD  $6.6^\circ$ ), which was significantly larger than that in the 87 subjects with no loss ( $20.1^\circ$ , SD  $13.8^\circ$ ;  $p = 0.0065$ , *t*-test). The MJSW in the eight AD patients with loss of copy number was 2.0 mm (SD 1.8 mm), which was not significantly different from that in the 87 patients with no loss (3.1 mm, SD 1.6 mm;  $p = 0.067$ , *t*-test). These nine AD cases all needed operative therapy, either pelvic osteotomy or total hip arthroplasty (Fig. 1b, Table III). Six of the nine AD cases had history of operative or conservative therapy for developmental dysplasia of the hip (DDH) (Table III).

## Discussion

A nationwide epidemiological study of hip OA in Japan showed that the relative frequency of AD among 485 hip OA patients was 361 (92.6%) in women and 29 (7.4%) in men of 390 AD patients,<sup>1</sup> which indicates that the aetiology of AD may differ between genders. Therefore, in the present study, we investigated the CNV of the *ASPN* gene only in female patients. Our data suggest that Sharp angle, CE angle, ARO angle, and MJSW are significantly worse in AD patients (Table I). All the radiological parameters of the hip in 95 subjects significantly correlated with each parameter (Table II). Sharp angle, CE angle, and ARO angle showed a strong correlation either positively or inversely with each other, which is in accordance with previous studies.<sup>26,27</sup> This means that each parameter could predict the other parameters, and suggests that Sharp angle, CE angle, and ARO angle are complementary for defining hip diseases such as AD and pincer-type femoroacetabular impingement.

Kizawa et al<sup>14</sup> previously identified *ASPN* as a susceptibility gene for knee and hip OA in the Japanese population. They detected seven alleles of the *ASPN* D repeat, containing D12 to D18 residues, and found that allele D14 was significantly more common in patients with OA, and allele D13 was significantly less common in OA patients compared with controls.<sup>14</sup> In the present study, there was a significant loss of copy number in the *ASPN* gene, including the *ASPN* D repeat region, of nine AD patients. Moreover, significantly worse radiological parameters were shown in the AD patients (Fig. 4). All nine cases needed operative therapy, and six of the nine cases had a history of therapy for DDH (Table III), indicating that loss of copy number within the region harbouring the *ASPN* gene on 9q22.31 was associated with severe AD. This is the first





**Fig. 2**

Fine structures of the loss of copy number in the 60 kb 9q22.31 region harbouring the *ASP*N gene resolved by Agilent high-density tiling microarray. Log<sub>2</sub> ratio (y-axis) was plotted using the moving mean along the genome position (x-axis). Plots for the nine affected acetabular dysplasia patients (cases 4, 7, 20, 25, 37, 39, 55, 59, and 61) are shown in parallel with plots of two control subjects (controls 19 and 26). The dark line represents a copy number plot along the genome for each subject between genomic positions 94 250 000 (left) and 94 310 000 (right). The orange areas indicate “loss of copy number”. The top map shows the positions of putative genes in the region.<sup>35</sup> *CEN*PP, centromere protein p; *ASP*N, asporin; *EC*M2, extracellular matrix protein 2.

report to demonstrate the association of the CNV of *ASP*N with AD.

CNVs or structural variations, such as a deletion or a gain of a genomic region, are increasingly being recognised as important inter-individual genetic variations across the human genome. Each variation could range in size from about 1000 nucleotide bases to several megabases.<sup>7</sup> As with other types of genetic variation, CNVs are significant contributors to human genetic disease and susceptibility. CNVs account for more nucleotide variation between two individuals than single-nucleotide polymorphisms.<sup>28,29</sup> The Agilent high-density custom-made array is a high-resolution array based on the comparative genomic hybridisation method, with 60 mer probes synthesised *in situ* as oligonucleotide arrays, at a spacing of 100 bp to 500 bp.<sup>8,17,30</sup> In the present study, the CNV region analysed on 9q22.31 and its flanking region contained the coding regions of the *ASP*N gene and the extracellular matrix protein 2 (*EC*M2) gene (Figs 2 and 3). This region contains multiple low-copy repeats in a small region of the genome, such as the *ASP*N D, which account for the instability of this region and trigger a copy number loss by an unequal crossing-over or end-joining event.<sup>31,32</sup> These characteristics suggest the possible involvement of a *de novo* occurrence of a loss of copy number in AD patients, and have mechanistic implications related to the sporadic nature of the disease. Although CNV was detected in the *EC*M2 gene in six patients (Figs 2 and 3), there have been no previous reports on skeletal phenotypes for the *EC*M2 gene. *EC*M2 is mainly expressed in adipose tissue as well as the mammary glands, ovaries, and uterus.<sup>33</sup> Moreover, *EC*M2 is related to B lymphopoiesis.<sup>34</sup> Hence, we consider that the *EC*M2 gene would not be a suitable candidate for risk susceptibility to skeletal disorders such as AD. On the other hand, gene(s) in this region may predispose to AD; of these genes, *ASP*N is considered a strong candidate based on previous reports.<sup>9-14</sup> We speculated that the *ASP*N gene in this region may be involved in the pathogenesis of AD through gene functional impairments caused by loss of copy number. Our findings indicate that decreased *ASP*N expression may lead to susceptibility to AD.

In conclusion, our study provides evidence for loss of genome copy number encompassing the *ASP*N gene region in nine AD patients. This *ASP*N loss may be an independent risk factor for severe AD of the hip. We analysed the AD case population using a high-density custom CNV array. As AD case populations were not large and the significance was not so high, more long-term studies should be conducted with larger sample numbers, including both genders in different ethnic groups for validation of the findings in the near future. Recent advances in DNA sequencing technology have further enabled the identification of CNVs by next-generation sequencing. Further studies on the potential relevance of

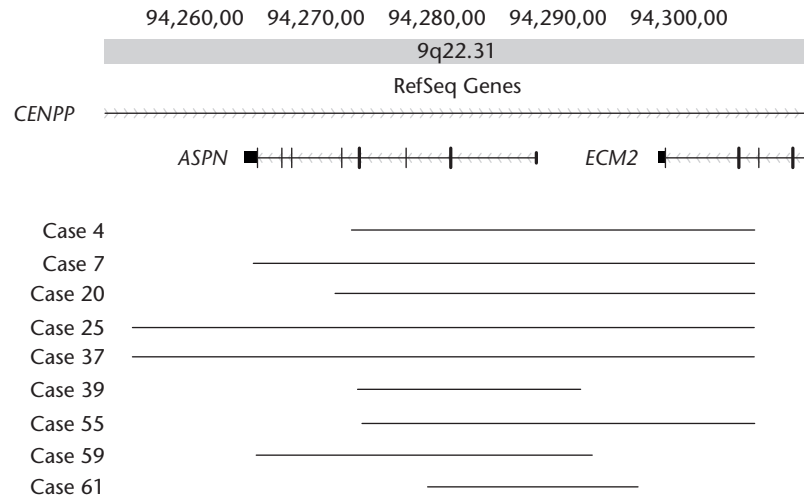


Fig. 3

Extent of the loss of copy number in the 60 kb region around the *ASPN* gene on 9q22.31 in the nine affected acetabular dysplasia patients. The horizontal bars represent the extent of the loss region in each case between the genomic positions 94 250 000 (left) and 94 310 000 (right). The top map shows the positions of putative genes in the region.<sup>35</sup> *CENPP*, centromere protein p; *ASPN*, asporin; *ECM2*, extracellular matrix protein 2.

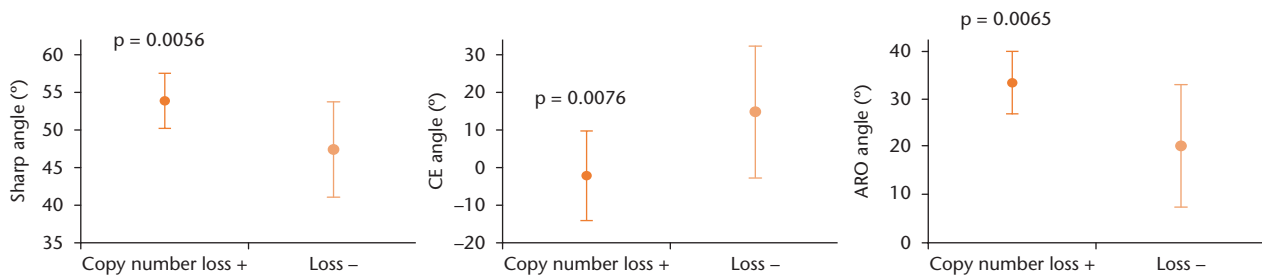


Fig. 4

Sharp angle, centre-edge (CE) angle, and acetabular roof obliquity (ARO) angle in acetabular dysplasia patients with positive loss of copy number in the *ASPN* gene compared with those in the 87 subjects with no loss in the *ASPN* gene. The vertical bar represents the extent of the radiological angle in each group. The orange round points indicate the mean radiological parameters, and the bars indicate the standard deviation.

**Table III.** Characteristics of the nine acetabular dysplasia patients with positive loss of copy number in the *ASPN* gene

| Case     | Age | Sharp angle | CE angle   | ARO angle | MJSW       | DDH | Surgical plan |
|----------|-----|-------------|------------|-----------|------------|-----|---------------|
|          | yrs | rt/lt (°)   | rt/lt (°)  | rt/lt (°) | rt/lt (mm) |     | rt/lt         |
| Case 4   | 59  | 50.0 / 49.8 | 14.1/15.2  | 28.5/23.4 | 2.4/3.5    | +   | PO / PO       |
| Case 7   | 51  | 49.1 / 45.1 | 8.7/19.1   | 30.1/15.2 | 1.9/4.5    | -   | PO / -        |
| Case 20  | 22  | 53.1 / 51.6 | 6.2/14.9   | 31.0/20.9 | 3.9/4.8    | -   | PO / PO       |
| Case 25  | 47  | 60.3 / 56.2 | -14.4/8.5  | 45.4/31.1 | 0/0        | +   | THA / THA     |
| Case 37  | 26  | 51.1 / 50.4 | 0/5.5      | 24.6/22.5 | 0/3.2      | -   | THA / PO      |
| Case 39* | 67  | 52.6 / 55.3 | -/-        | 30.5/31.3 | -/-        | +   | THA / THA     |
| Case 55  | 69  | 51.7 / 51.2 | -19.5/-7.4 | 40.7/37.5 | 0/3.0      | +   | THA / PO      |
| Case 59  | 45  | 52.3 / 55.4 | -5.4/-16.9 | 35.7/36.9 | 4.5/3.7    | +   | PO / PO       |
| Case 61  | 42  | 56.3 / 52.7 | 3.5/9.5    | 29.6/22.2 | 3.7/3.5    | +   | PO / PO       |

\*Case 39 had a bilaterally high congenital dislocation of the hip

†CE, centre edge; ARO, acetabular roof obliquity; MJSW, minimum joint space width; DDH, developmental dysplasia of the hip; rt, right side; lt, left side; PO, pelvic osteotomy; THA, total hip arthroplasty

*ASPN* function to AD will contribute not only to the determination of the pathogenesis of hip OA, but also to the identification of a novel therapeutic target for the disease. The CNV of *ASPN* could be a predictive tool to identify severe AD patients, and potentially delay or prevent the onset of end-stage OA of the hip by screening patients at

a young age. Further study is required, however, in future.

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#### Author Contributions:

- T. Sekimoto: Study design, Data collection and analysis, Writing the paper.
- M. Ishii: Data collection and analysis, Editorial contribution.
- M. Emi: Study design, Data collection and analysis, Editorial contribution.
- S. Kurogi: Data collection.
- T. Funamoto: Data collection.
- Y. Yonezawa: Statistical analysis.
- T. Tajima: Data collection.
- T. Sakamoto: Data collection.
- H. Hamada: Data collection.
- E. Chosa: Editorial contribution.

#### Associated Societies:

- Japanese Orthopaedic Association, Japanese Hip Society.

#### Conflicts of Interest Statement

- None declared

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